ELISA (Enzyme-Linked Immunosorbent Assay)

(Wright *et al.*, 1987 & Wright *et al*, 1996)

INTRODUCTION

This assay allows you to determine the concentration of glomalin, specifically. It uses two antibodies, one which is conjugated to biotin with a long spacer arm which gives higher specificity. This procedure also allows you to use a very tight curve and get very exact concentration values. It is important to note that Dynex U-shaped microtiter plates should be used (see inventory for ordering info). The shape of the well and type of plastic makes a difference in the specificity. The amount of sample needed is determined by using the Bio-Rad Bradford Protein Assay and calculating for a concentration between 0.01-0.03 ug/well.

MATERIALS

Dynex 96 well microtiter plate
tilt table or shaker
plate reader with a 405 or 410 filter
2% non-fat milk (2g powdered milk/100ml of PBS)
PBS (Phosphate buffered saline)
PBST (PBS with 0.2 ml/L Tween 20)
1% BSA (1 g BSA/100 mls PBS) (Make about 500-1000 mls of stock and dispense in 6 ml aliquots that will be frozen until needed.)
MAb32B11 antibody (Dilute 1 ml antibody in 5 mls PBS))
Biotinylated anti-mouse IgM (1:2500 in 1% BSA) (4.8µl/6ml)
ExtrAvidin peroxidase (1:2000 in 1% BSA) (3µl/6ml)
color devloper (7.5 ml/plate)

Standards and the standard curve

1. Make up a stock solution of 0.08 ug of protein/100 ul of PBS and store at **4^{o}C** (Do not freeze the ELISA stock solution or use it if it appears to be contaminated or is several months old.)

   To make the stock:

   1. Use protein extracted from fresh hyphae that is nearly 100% immunoreactive. To determine this, run a Bradford and an ELISA assay on the samples and compare concentrations.

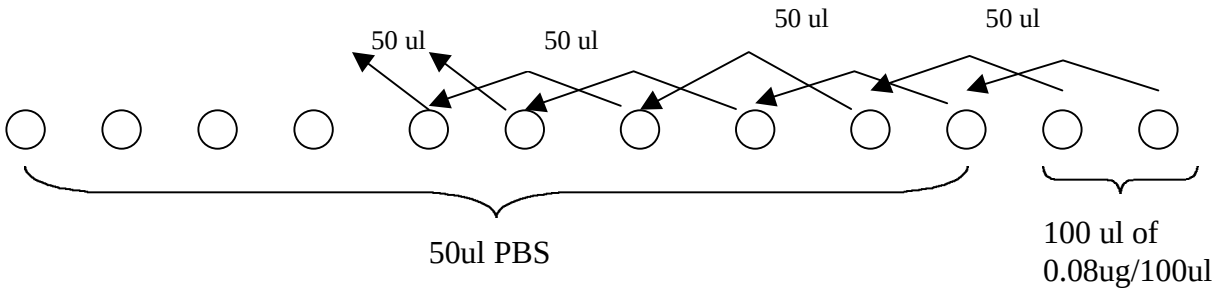
   2. If Bradford and ELISA values are nearly the same, make an ELISA curve and test the values by comparing results to a known curve.

   3. Make up 500 ul aliquots of the stock with a concentration of 0.08 ug of protein in 100 ul of PBS or 0.40 ug of protein in 500 ul of PBS.
2. Put 100 ul of the 0.08 ug protein/100 ul of PBS in 2 of the wells and 50 ul PBS in the other 10 wells.
3. Transfer 50 ul of the 0.08 ug sample to a neighboring well that has 50 ul PBS.
4. Mix 3-4 times with the micropipet by pulling the sample up and down.
5. Remove 50 ul from these 2 wells and transfer to 2 neighboring wells. Mix 3-4x. Repeat for the these 2 wells.
6. After the 3rd dilution, remove 50 ul from the 2 wells that have 100 ul and dispose of it.

Wells will have the concentrations or be diluted as outlined below:

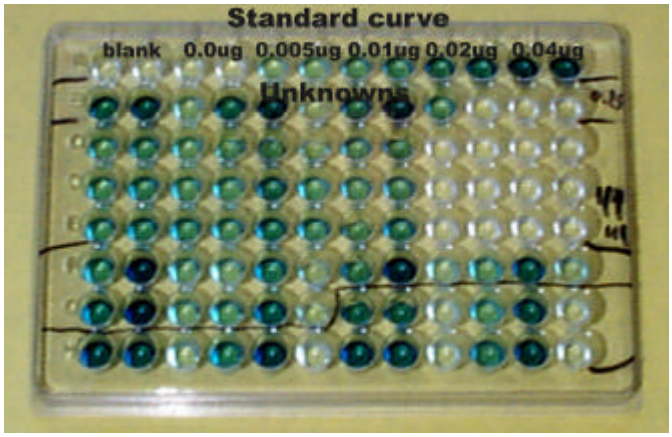
Well #	Protein concentration (ug/50 ul)
1	0
2	0
3	0
4	0
5	0.005
6	0.005
7	0.01
8	0.01
9	0.02
10	0.02
11	0.04
12	0.04

Row of wells on the ELISA plate



METHODS

1. Add Bradford calculated amount of sample plus enough PBS to equal a total of 50 ul or 50ul of pre-diluted sample to a well (see calculations sheet).
2. Let it dry over night (make sure that it is completely dry).
3. Add 250 ul/well of freshly prepared 2% non-fat milk to wells and incubate on shaker for 15 min. Flip plate into sink to remove milk and blot (via inverting and hard taps) on an absorbent paper towel.
4. Add 50ul/well of diluted MAb 32B11antibody and incubate on shaker for 1 hr. Flip plate to remove and blot with paper towel (via inverting and hard taps). Wash with PBST 3x, blotting between washes.
5. Add 50ul/well of biotinylated IgM antibody, diluted in 1% BSA, and incubate on shaker for 1 hour. Flip plate to remove and blot with paper towel (via inverting and hard taps). Wash with PBST 3x, blotting between washes.
6. Add 50ul/well of ExtrAvidin peroxidase***, diluted in 1% BSA, and incubate for 1 hour on shaker. Flip plate to remove and blot with paper towel. Wash with PBST 4x, blotting between washes.
7. If you are making the color developer by hand and not from the kit, begin working on this about 20-30 min before the ExtrAvidin peroxidase incubation is completed. Start by checking and adjusting the pH of the citric acid (pH 4.0). Then, carefully weigh out the ABTS powder and dissolve it in dH_2O. About 5 min before the ExtrAvidin peroxidase incubation is completed, measure out the citric acid and add the ABTS solution. **DO NOT** add the hydrogen peroxide until after the PBST washings (right before use). If you are using the Bio-Rad horseradish peroxidase kit, **DO NOT** mix the solutions together until right before use (after the PBST washings). For either method, if you are doing more than one plate at a time, the color developer should be made for each plate individually.
8. Add 50ul/well of color developer** (must be quick and accurate).
9. After addition of color developer, incubate 15 minutes and read at 405 nm with plate reader. Determine concentration following the equations outlined on the calculations sheet.



*Color developer

Note: Instead of mixing these solutions individually and because the ABTS may not always be fresh by the time of delivery, a kit may be purchased from Bio-Rad that is stabilized for a year.)

Just before use mix:

Citrate buffer	ABTS soln.	30% H2O2
10 ml	200 ul	10 ul
7.5 ml	150 ul	7.5 ul
5 ml	100 ul	5 ul
2.5 ml	50 ul	2.5 ul

Citrate buffer: For 100 ml use 1.05 g of citric acid (not sodium citrate) and adjust the pH to 4.0 using 6N and 2N NaOH. **Check pH before each use.**

ABTS solution: 0.015 g 2,2'-azino-di-(3-ethylbenzthiaoline sulfonic acid)/1 ml H2O (Must be fresh = doesn't turn a strong green color in water)

Hydrogen peroxide: To test freshness: A 1:1000 dilution of 30% H2O@ in PBS should have an optical density of ~0.7 at 230nm (see p. 150 of Goding 2nd Ed. Monoclonal Antibodies: Principles and Practices)

ELISA ranges for OD values for the standard curve

Typical

Concentration (ug/well)	OD value
0	0
0.05	0.200-0.450
0.1	0.550-0.750
0.2	1.000-1.250
0.4	1.650-2.000

Sometimes the curve will run this high

Concentration (ug/well)	OD value
0	0
0.05	0.200-0.400
0.1	0.600-0.750
0.2	1.700-2.000
0.4	2.700-3.000

Note: Because this curve has a slight quadratic shape, it is advised to dilute samples at the top of the curve in half and recheck them. It is also advised to rerun the sample at the bottom of the curve at 2x the concentration.

***An alternative method is currently being applied in our lab where ExtrAvidin Alkaline Phosphatase is being used rather than the ExtrAvidin Peroxidase. This method has the potential for enhanced color development and is more stable because hydrogen peroxide is not being used.

ExtrAvidin Alkaline Phophatase Method

(1) Follow steps 1-5 as outlined in the methods procedure above.

(2) Dilute the ExtrAvidin Alkaline Phosphatase in 1% BSA (3ul/6mls) and add 50ul of the diluted solution to each well.

(3) Incubate for 1 hr. After incubation, flip plate to remove and blot with paper towel. Wash with PBST 3x, blotting between washes.

(4) The fourth wash should be done with TBST not PBST, because PBST will react with the phosphatase enzyme. (TBST is Tris Buffered Saline (250mM NaCl, 10mM Tris(hydroxymethyl)aminomethane, and 0.2 ml/L of Tween 20) at pH 7.4).

(5) Dissolve one tablet of Sigma 104 Phosphatase Substrate (5mg tablets) in 5 mls of DEA buffer (Mix 97 ml of diethanolamine buffer, 10%, with 1L of 0.01% MgCl2 solution, and adjust the pH to 9.8 with 1 N HCl. The solution must be kept sterile and stored covered at room temperature).

(6) Add 50ul to each well and incubate for 30 min. Read plate at 405 or 410 nm.